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Hydrophobically Modified Chitosan Gauze for Control of Massive Hemorrhage

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14. ABSTRACT

Currently, the standard of care for treating severe hemorrhage in a military setting is Combat Gauze™ (CG). Previous work has shown that hydrophobically modified (hm) chitosan has great hemostatic potential. This work aims to create an hm chitosan-coated gauze to compare to CG as well as ChitoGauze (ChG) in an in vivo hemorrhage model. Twelve Yorkshire swine were randomized to receive either hm chitosan gauze (n = 4), ChG (n = 4), or CG (n = 4). We found hm chitosan gauze to be at least equivalent to CG and ChG in terms of overall survival (100% vs. 75%), number of dressings used (6 vs. 7), and duration of hemostasis (3 hours vs. 2.25 hours). Total post-treatment blood loss was lower in the hm chitosan gauze treatment group (4.7 mL/kg) when compared to the CG (13.4 mL/kg) and ChG (12.1 mL/kg) groups. In a lethal hemorrhage model, hm chitosan gauze appears to have outperformed both CG and ChG. However, given the small treatment group size, the only measured outcome that was significantly different was total post-treatment blood loss. Future work will be performed on a hypothermic and coagulopathic model that should allow for outcome significance to be differentiated under small treatment groups.

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1.0 SUMMARY

Currently, the standard of care for treating severe hemorrhage in a military setting is Combat Gauze™ (CG). Previous work has shown that hydrophobically modified (hm) chitosan has great hemostatic potential. This work aims to create an hm chitosan-coated gauze to directly compare to CG as well as ChitoGauze® (ChG) in a lethal in vivo hemorrhage model.

Twelve Yorkshire swine were randomized to receive either hm chitosan gauze (n = 4), ChG (n = 4), or CG (n = 4). A standard hemorrhage model was used in which animals underwent a splenectomy prior to a 6-mm punch arterial puncture of the femoral artery. Thirty seconds of free bleeding was allowed before dressings were applied and compressed for 3 minutes. Baseline mean arterial pressure was preserved via fluid resuscitation. Experiments were conducted for 3 hours, after which any surviving animal was euthanized.

Hydrophobically modified chitosan gauze was found to be at least equivalent to both CG and ChG in terms of overall survival (100% vs. 75%), number of dressings used (6 vs. 7), and duration of hemostasis (3 hours vs. 2.25 hours). Total post-treatment blood loss was lower in the hm chitosan gauze treatment group (4.7 mL/kg) when compared to the CG (13.4 mL/kg) and ChG (12.1 mL/kg) groups.

Hydrophobically modified chitosan gauze appears to have outperformed both CG and ChG in a lethal hemorrhage model. However, given the small treatment group size, the only measured outcome that was significantly different was total post-treatment blood loss. Future comparison of hm chitosan gauze to CG and ChG will be performed on a hypothermic and coagulopathic model that should allow for outcome significance to be differentiated under small treatment groups.

2.0 INTRODUCTION

Trauma deaths are spread indiscriminantly across racial and economic backgrounds [1]. In the United States, trauma injuries are responsible for more years of life lost than heart disease and cancer combined [2]. Hemorrhage has been, and still is, a major cause of mortality from trauma injuries in both the civilian and military setting [3-7]. Despite these mortality rates from hemorrhage, little advancement was made on cellulosic gauze, which was standard treatment for over 2,000 years [8]. However, much effort has been put into developing advanced hemostats over the past 15 years. First-generation advanced hemostats, Quickclot® Powder and Hemcon Bandage®, had issues with toxicity [9,10] and tissue adhesion, respectively [11,12]. These gave way to a second generation of advanced hemostats, which included Woundstat™, the FAST dressing, and Quickclot® Combat Gauze™ (CG). Of this second generation, CG has proven quite effective in treating severe hemorrhage and has become standard of care for use in the U.S. military [13].

Ideally, an advanced hemostat for topical use in treating severe hemorrhage would be both inexpensive and able to treat the highest mortality patients who suffer from coagulopathy and hypothermia. The FAST dressing was demonstrated to be quite effective in treating coagulopathic and hypothermic swine in a severe hemorrhage model [14]. However, as is the case with all other fibrinogen-based hemostats, the FAST dressing will likely be significantly more expensive than competing non-fibrinogen-based tropical hemostats. In the same study that illustrated the FAST dressing's effectiveness in a coagulopathic and hypothermic swine model, CG was shown to have limited effectiveness under these conditions [14]. With all this in mind,

there obviously still remains room for development of a third generation of advanced hemostats that would be both effective in treating the highest mortality hemorrhages and also cost competitive with other topical hemostats.

3.0 BACKGROUND

Hydrophobically modified (hm) chitosan and alginate have been shown to promote hemostasis, decrease blood loss, and increase survival in lethal animal models [15-17]. The mechanism of action for the hemostatic capability of hm chitosan and alginate is the formation of non-biological clots arising from hydrophobes interacting with the cell membrane, thereby utilizing blood cells as crosslinks in the formation of a polymer matrix. In addition, hydrophobic modification of both chitosan and alginate increases tissue adhesion, which is thought to further enhance both hm polymers' ability to act as a hemostatic dressing.

Hydrophobically modified chitosan has previously been utilized in the form of both a pad and foam for treating lethal hemorrhages in animal models [15,17]. We developed hm chitosan into usable and effective gauze, which is the most common form of hemostatic dressing encountered by a first responder. Our study was a head-to-head comparison of hm chitosan gauze with CG and ChitoGauze® (ChG) in terms of clotting capability, tissue adhesion, and effectiveness in a swine hemorrhage model. In addition, we demonstrated the ability of hm chitosan to clot/gel diluted blood, which is representative of coagulopathic patients. Overall this study has shown hm chitosan gauze to be at least as effective as, and possibly better than, CG or ChG in a lethal hemorrhage model.

4.0 METHODS

4.1 Materials

Chitosan (molecular weight 190–310K) and *n*-dodecyl aldehyde were obtained from Sigma-Aldrich (St. Louis, MO). Band-Aid First Aid Covers Kling Rolled Gauze, ChitoGauze®, Combat Gauze™, and Woundstat™ were purchased from Johnson & Johnson (New Brunswick, NJ), HemCon Medical Technologies (Portland, OR), Z-Medica (Wallingford, CT), and TraumaCure (Bethesda, MD) respectively. L-929 mouse fibroblast cells were purchased from American Type Culture Collection (Manassas, VA). Adult bovine whole blood with sodium citrate was purchased from Lampire Biological Laboratories (Pipersville, PA). Lactated Ringer's Injection USP was obtained from Baxter (Deerfield, IL). Eagle's minimal essential medium (EMEM), fetal bovine serum, penicillin, and streptomycin were obtained from ThermoScientific (Waltham, MA). Live/Dead® assay kit for mammalian cells was purchased from Invitrogen (Grand Island, NY).

4.2 Hydrophobically Modified Chitosan Synthesis and Preparation of hm Chitosan Gauze

Hydrophobically modified chitosan was synthesized as previously described. For gauze preparation, 2 wt% solutions of hm chitosan were then made. Band-Aid First Aid Covers Kling Rolled Gauze was then soaked in this hm chitosan solution for 2 hours. Subsequently, excess hm chitosan solution was removed and hm chitosan gauze was allowed to air dry for 12 hours. After

air drying, hm chitosan gauze was Z-folded and vacuum sealed in airtight packaging. Hydrophobically modified chitosan gauzes were then sterilized via gamma irradiation at a dose range of 25-40 kGy at Steris Corporation. Figure 1 below shows a picture of the hm chitosan gauze.

Figure 1. Photograph of Z-folded hm chitosan gauze.

4.3 Diluted Blood Gelation

A 50/50 solution of bovine heparinized blood and Lactated Ringer's Injection USP was made. Then, this 50/50 solution was mixed with a 1 wt% solution of hm chitosan or chitosan at a ratio of 1 to 2. After vortexing the mixtures for 30 seconds, the vials were inverted to test for gelation. Similar procedures were followed in which Lactated Ringer's Injection USP was replaced with either normal saline or Hextend.

4.4 Thromboelastography

Thromboelastography (TEG) was performed with Woundstat^{TM}, CG, ChG, and hm chitosan gauze in a similar manner as previously described [18].

4.5 Biocompatibility Studies

L-929 mouse fibroblast cells were seeded into a 24-well plate at 70,000 cells per well and allowed to grow for 48 hours in complete EMEM (incomplete EMEM + 5% fetal bovine serum with 100 IU/mL penicillin and 100 μg/mL streptomycin). Extracts of hm chitosan gauze were prepared by first testing the absorption of the bandages. This was achieved by contacting samples with 50 mL incomplete EMEM per gram of sample for 24 hours at 37°C and 60 rpm in a glass vial. After 24 hours, the volume of the remaining fluid was measured and used to determine the amount of fluid absorbed per gram of sample (mL/g). After sample absorption is determined, 1 gram of sample was contacted with complete EMEM in a glass vial at a ratio 5 mL/g greater than the calculated absorption. Extracts were then incubated at 37°C and 60 rpm for 24 hours. Extracts were then tested on previously seeded L-929 mouse fibroblast cells. Each well on the plate was aspirated and replaced with 1.0 mL of extract. The cells were then incubated with the extracts for 72 hours at 37°C in humid air with 5% carbon dioxide. Cells

were then imaged and observed under 100x optical magnification on a confocal microscope (Leica SP5 X). Cells were then washed once with 1.0 mL phosphate-buffered saline per well and contacted with 1.0 mL phosphate-buffered saline containing 4 μM calcein AM (live stain) and 4 μM ethidium homodimer (dead stain). Each well was then imaged at 100x using a green fluorescent protein fluorescence filter (ex:473 em:520) to observe the live stain and a Texas red fluorescence filter (ex:562 em:624) to observe the dead stain. Fluorescent images were then overlaid to create a composite live-dead image.

4.6 Tissue Adhesion Studies

Tissue adhesion studies were conducted on CG, ChG, and hm chitosan gauze in the same manner as described by DeCastro et al. [15].

4.7 Surgical Preparation, Instrumentation, Procedures

Twelve female Yorkshire pigs, weighing 37.2 ± 2.2 kg, were obtained from the Thomas D. Morris Institute of Surgical Research (Reisterstown, MD). All animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine. The swine were prepared, anesthetized, incubated, placed on mechanical ventilation, and maintained as described in DeCastro et al. [15].

Surgical procedures were performed as previously described [15], except that a 6.0-mm-diameter vascular punch was used instead of a 4.4-mm-diameter punch. Animal survival was defined as partial pressure of carbon dioxide greater than 20 mmHg after 180 minutes. Any surviving animals at the end of the study period were euthanized with 100-200 mg/kg intravenous pentobarbital.

4.8 Data Analysis

Data are expressed as mean ± standard deviation (SD) and analyzed by analysis of variance (paired t-test), Fisher's exact, and log-rank for statistical comparisons. All p-values were adjusted according to the false discovery rate method for bi-group comparison. Data with high variance were log transformed for analysis of variance. Statistical significance was assigned at a greater than 95% confidence level ($p < 0.05$).

5.0 RESULTS

5.1 In Vitro

To demonstrate the ability of hm chitosan to form strong, non-biologic clots even under coagulopathic conditions, hm chitosan at 1 wt% was added with 50/50 mixtures of blood with Hextend. All three mixture formed gels that were able to hold their own weight when inverted, which suggests the formation of a strong gel (Figure 2). Additional TEG studies were undertaken to look at the ability of the hm chitosan gauze to activate the natural clotting cascade.

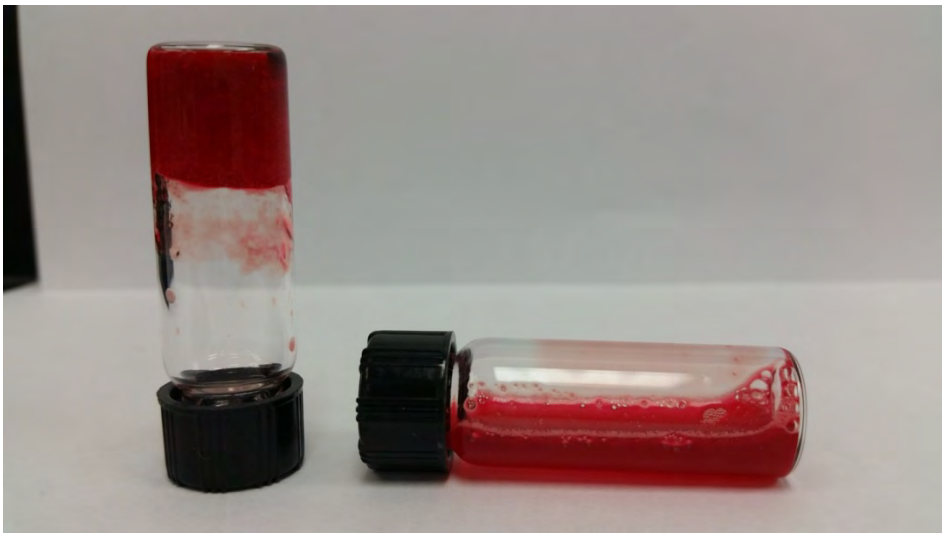

Figure 2. Photograph of hm chitosan (0.6 wt%) (left) and chitosan (0.6 wt%) (right) mixed with Hextend-diluted blood.

As shown in Table 1, the blood clotting activities of both the hm chitosan gauze and ChG were decreased when compared to that of Woundstat™ and CG. This is not totally unexpected, since chitosan-based products are thought to be effective at stopping hemorrhaging due to chitosan's muco-adhesive properties, not through any inherent hemostatic capability, unlike CG and Woundstat™, which accelerate the clotting process by concentrating clotting factors.

Table 1. TEG Parameter Summary Results

Dressing Type	R (min)	K (min)	Angle (deg)	LY 30 (%)	A (mm)
Woundstat	4.5 ± 0.3	2.3 ± 0.2	61.3 ± 2.6	0.9 ± 0.2	72.6 ± 1.3
Combat Gauze	4.0 ± 0.2	2.1 ± 0.2	65.1 ± 3.3	1.0 ± 0.2	71.1 ± 2.0
ChitoGauze	6.8 ± 0.3	3.7 ± 0.3	50.2 ± 2.7	0.8 ± 0.1	72.5 ± 1.8
hm Chitosan Gauze	6.5 ± 0.2	3.5 ± 0.2	55.1 ± 2.3	0.9 ± 0.2	73.1 ± 1.9

Additionally, all gauzes examined in this work were subjected to tissue adhesion experiments. Figure 3 shows the results of these studies. Both chitosan-based gauzes were more adhesive than CG. This is not unexpected due the different modes of hemostatic action between CG and chitosan-based products. Furthermore, hm chitosan gauze was significantly more tissue adherent than ChG (Figure 3).

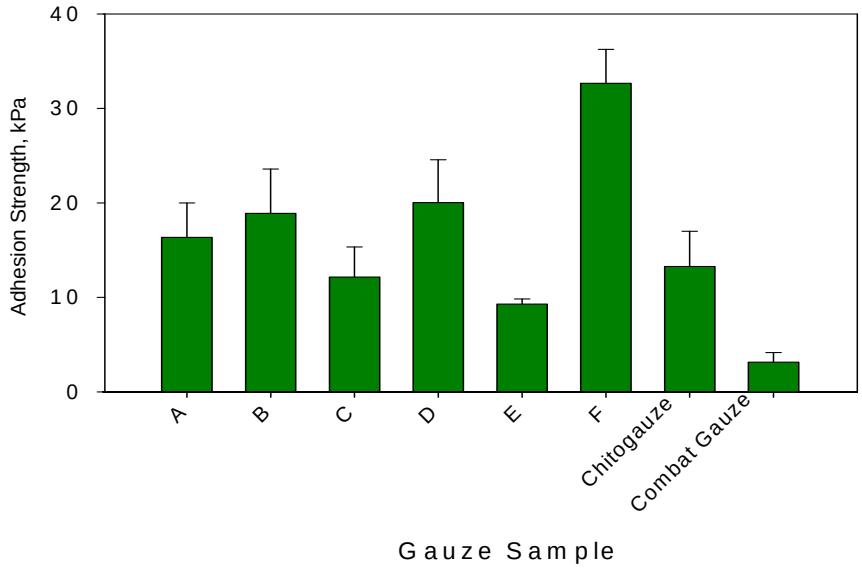

Figure 3. Histogram of tissue adhesion strength hm chitosan gauze samples relative to ChitoGauze and Combat Gauze.

Initial qualitative biocompatibility studies were undertaken. These experiments took the form of live-dead assays on L-929 mouse fibroblast cells that had been incubated in extracts of hm chitosan gauze. These qualitative studies showed no significant cell death from visual inspection. (Figure 4).

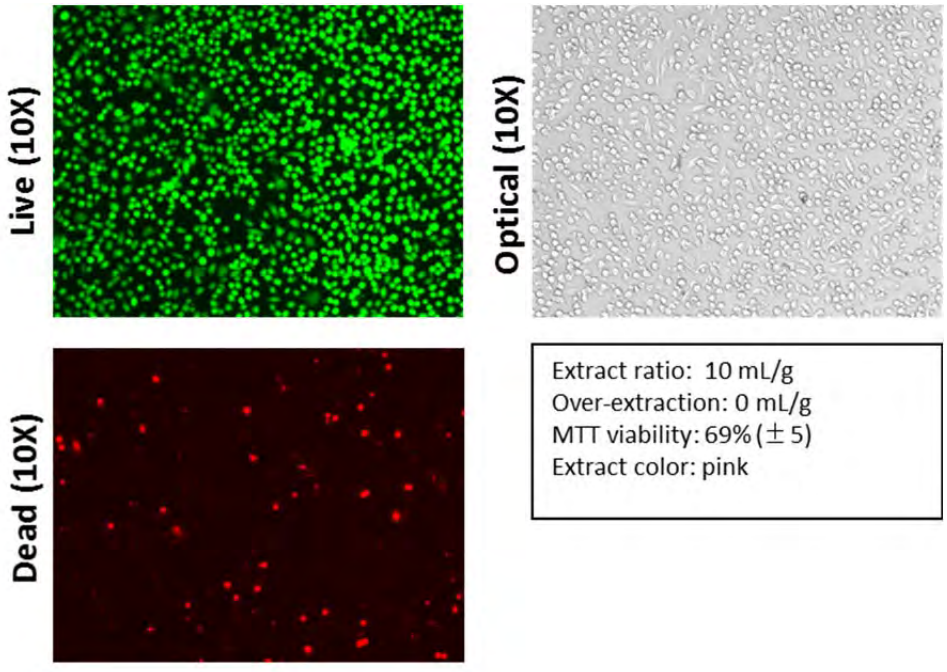

Figure 4. Cytotoxicity of hm chitosan gauze.
Left images show live-dead stains of hm chitosan gauze extracts after 72 hours. Right image shows optical transmission of cells after 72 hours. Bottom right corner displays the extraction ratio used, as well as MTT viability of cells after 72 hours.

5.2 In Vivo

Table 2 illustrates the baseline parameters and characteristics of the animals used. After these animals' characteristics were measured, the animals were randomly assigned to treatment groups.

Table 2. Baseline Parameters and Animal Characteristics

Variable	Mean ± SD
Body Weight, kg	37.2 ± 2.2
Body Temperature, °C	37.15 ± 0.44
Hematocrit, %	30.4 ± 1.3
Hemoglobin, g/dL	11.04 ± 0.58
Platelets, 1000/μL	311 ± 53
Prothrombin Time, s	9.4 ± 0.5
Activated Partial Thromboplastin Time, s	15.3 ± 1.06
Fibrinogen, mg/dL	216 ± 52
pH	7.44 ± 0.03
Pre-Injury Mean Arterial Pressure, mmHg	69.2 ± 8.1

Table 3 summarizes the outcomes of these in vivo experiments. Control groups (n = 4) of both CG and ChG were able to achieve initial hemostasis in 75% of animals using a total of seven bandages. Performing slightly better, the hm chitosan gauze was able to achieve hemostasis in 100% of animals while using only six bandages. In addition, duration of hemostasis was longer for the hm chitosan group (3 hours) when compared to CG (2.25 hours) and ChG (2.25 hours). While overall survival, duration of hemostasis, and number of bandage used are not significantly different among treatment groups, post-treatment blood loss is significantly lower for the hm chitosan gauze group when compared to other treatment groups.

Table 3. Outcomes for Treatment of a Severe Arterial Hemorrhage with Different Hemostatic Dressings in Swine

Dressing Type	No. of Animals	No. of Dressings Used	Pre-Treatment Blood Loss (mL/kg)	% Initial Hemostatis Achieved[a]	Post-Treatment Blood Loss (mL/kg)	Duration of Hemostasis (h)	Survival Time (h)
Combat Gauze	4	7	7.9 ± 2.1	75 (3/4)	13.4 ± 15.1	2.25 ± 1.5	2.25 ± 15
ChitoGauze	4	7	8.3 ± 2.5	75 (3/4)[b]	12.1 ± 13.3[c]	2.25 ± 1.5[d]	2.25 ± 1.5[d]
hm Chitosan Gauze	4	6	7.7 ± 1.7	100 (4/4)[e]	4.7 ± 3.1[f]	3[g]	3[g]

Note: Data expressed as mean ± SD.
[a]Initial hemostasis was considered to occur after bleeding stopped for at least 3 minutes after compression.
[b]vs. Combat Gauze, not significant (NS) (Fisher's exact test).
[c]vs. Combat Gauze, NS (paired t-test).
[d]vs. Combat Gauze, NS (log-rank test).
[e]vs. Combat Gauze, NS, vs. ChitoGauze, NS (Fisher's exact test).
[f]vs. Combat Gauze, NS, vs. ChitoGauze, NS (paired t-test).
[g]vs. Combat Gauze, NS, vs. ChitoGauze, NS (log-rank test).

Figure 5 is a Kaplan-Meier curve of survival data. This figure shows that all animals in the hm chitosan treatment group survived for the entire 3-hour duration of the experiments while only three out of four survived for the control groups of CG and ChG.

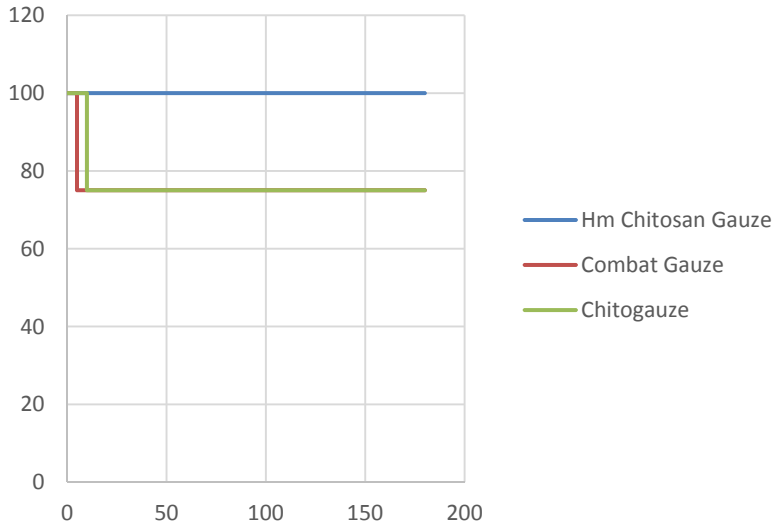

Figure 5. Kaplan-Meier analysis of survival data.

6.0 DISCUSSION

This work utilizes a lethal arterial injury model in swine to evaluate the hemostatic capability of three gauzes: CG, ChG, and hm chitosan gauze. CG was chosen as a point of comparison because it currently is the current standard of care for hemorrhage control in the military setting [13]. In addition to CG, ChG, a gauze coated with unmodified chitosan, was chosen as another hemostatic product to compare to hm chitosan gauze. Table 3 and Figure 5 illustrate the outcomes of these animal model experiments. While not statistically significant, the hm chitosan gauze outperformed both CG and ChG in terms of overall survival, number of dressings used, and duration of hemostasis. Interestingly, post-treatment blood loss was significantly lower in the hm chitosan gauze group when compared the two other gauzes. Post-treatment blood loss has been shown to correlate with survival in other similar studies [14,15]. Given this correlation, one could speculate that if a larger group of animals was studied, hm chitosan gauze's outperformance of CG and ChG would be significant.

In addition to in vivo studies, this work undertakes a number of in vitro studies that serve to demonstrate both the increased hemostatic capabilities of hm chitosan gauze and the difference in in hemostatic action between chitosan-based and mineral-based hemostatic products. Chitosan-based products work by strongly adhering to surrounding tissue, which plugs the wound in a similar fashion to a beaver dam stopping the flow of water in a stream. This mechanism is supported by Figure 3, which shows that both ChG and hm chitosan gauze have dramatically increased tissue adhesion strength when compared to CG. It has been speculated that one of the reasons hm chitosan itself has consistently outperformed native chitosan in terms of hemostatic capability is increased tissue adhesion. Tellingly in this study, the hm chitosan gauze is significantly more tissue adherent than ChG, which suggests a superiority in hemostatic

potential of the hm chitosan gauze. Unlike chitosan-based hemostats, mineral-based products work by absorbing blood, which concentrates clotting factors and facilitates the activation of the natural clotting cascade. This mechanism is supported by Table 1, which illustrates the TEG results on extracts of hm chitosan gauze, ChG, CG, and Woundstat™. As seen in this table, both CG and Woundstat™ demonstrate enhancement of the blood's natural clotting cascade when compared to the hm chitosan gauze and ChG. Overall, both Figure e and Table 1 show differences in hemostatic action between chitosan-based and mineral-based hemostatic products.

Early attempts at creating advanced hemostatic products resulted in some products, such as Quickclot® powder and Woundstat™, causing adverse side effects [9,10,19]. For this reason, initial biocompatibility studies were conducted on the hm chitosan gauze. Figure 4 shows no significant cell death when extracts of hm chitosan gauze were exposed to L-929 mouse fibroblast cells.

7.0 CONCLUSIONS

This work has shown that hm chitosan gauze was equivalent to or better than CG and ChG in performance in treating a lethal hemorrhage model. In this model, hm chitosan gauze significantly lowered post-treatment blood loss, suggesting that in a larger study hm chitosan gauze may significantly improve overall survival. Initial data demonstrate hm chitosan can gel diluted blood (Figure 2), which indicates hm chitosan gauze may be effective in treating coagulopathic patients. To further demonstrate the superiority of hm chitosan gauze over CG and ChG, future studies will be conducted on a hypothermic and coagulopathic hemorrhage model that has shown CG to be ineffective.

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LIST OF ABBREVIATIONS AND ACRONYMS

CG	Combat Gauze™
ChG	ChitoGauze®
EMEM	Eagle's minimal essential medium
hm	hydrophobically modified
NS	not significant
SD	standard deviation
TEG	thromboelastography